

Characterization and selection of inter simple sequence repeat markers for genetic studies of mesquite

Caracterização e seleção de marcadores inter simple sequence repeat para estudos genéticos de algaroba

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Abstract

Since characterization and selection of molecular markers are crucial steps in genetic research for plant conservation and/or (pre) breeding, this study aimed to describe the amplification profile of Inter Simple Sequence Repeat (ISSR) markers in *Prosopis juliflora* (Sw.) DC. so that the most suitable ones could be selected. To do so, the genomic DNA of 16 individuals collected at the Federal Institute - Campus Itapetinga, Bahia State, were extracted at the Applied Molecular Genetics Laboratory of the State University of Southwest Bahia, in Itapetinga-BA (Brazil). The amplification profile was obtained using 23 ISSR primers, visualized after electrophoresis in 2% agarose gels, and photo-documented under ultraviolet light; then, genotyping was performed. In total, amplification of 206 markers was observed, with an average of nine tags per primer, a polymorphic percentage equivalent to 90% of the markers generated; and mean polymorphic information content of 0.63. Expected and observed heterozygosity means were similar (0.28 and 0.29, respectively). Our findings confirm that ISSR primers are suitable for molecular-genetic studies of *P. juliflora*. Among the 23 analyzed primers, 13 can be prioritized (DiGA3'G, DiCA 3'RG, DiCA 3'YG, DiGA 3'C, DiGA 3'RC, DiGA 3'T, TriCAG 3'RC, TriTGT 3'YC, TriAAC 3'RC, TriAAG 3'RC, TriCGC 3'RC, TriGAC 3'RC, and TriGGA 3'RC) for genetic studies of *P. juliflora*.

Keywords: ISSR; Polymorphism; *Prosopis juliflora*; Transferability.

Resumo

Considerando que a caracterização e seleção de marcadores moleculares é uma etapa importante para subsidiar pesquisas genéticas dedicadas a conservação e, ou, (pré) melhoramento, o presente estudo caracterizou o perfil de amplificação de marcadores *Inter Simple Sequence Repeat* (ISSR) em *Prosopis Juliflora* (Sw.) DC. e selecionou

aqueles que apresentaram perfil mais adequado. Para tanto, foram extraídos no Laboratório de Genética e Molecular Aplicada, da Universidade Estadual do Sudoeste da Bahia, em Itapetinga-BA, o DNA genômico de 16 indivíduos coletados no Instituto Federal, Campus Itapetinga, Bahia. O perfil de amplificação foi obtido com uso de 23 iniciadores ISSR, visualizado após a eletroforese em géis de agarose a 2%, fotodocumentado com incidência de luz ultravioleta e, em seguida, foi realizada a etapa de genotipagem. No total foram observadas amplificação de 206 marcadores com média de nove marcas por iniciador, percentual polimórfico equivalente a 90% dos marcadores gerados; conteúdo de informação polimórfica médio de 0,63. Os valores médios de heterozigosidade esperada e observada foram semelhantes, com 0,28 e 0,29, respectivamente. Os resultados confirmam que os iniciadores ISSR possuem características adequados para estudos genético-moleculares em *P. juliflora*, sendo possível destacar entre os 23 iniciadores, 13 a serem priorizados (DiGA3'G, DiCA 3'RG, DiCA 3'YG, DiGA 3'C, DiGA 3'RC, DiGA 3'T, TriCAG 3'RC, TriTGT 3'YC, TriAAC 3'RC, TriAAG 3'RC, TriCGC 3'RC, TriGAC 3'RC e TriGGA 3'RC) em estudos genético de *P. juliflora*.

Palavras-chave: ISSR; Polimorfismo; *Prosopis juliflora*; Transferabilidade.

Resumen

Considerando que la caracterización y selección de marcadores moleculares es un paso importante para apoyar la investigación genética dedicada a la conservación y, o, (pre) mejora, el presente estudio caracterizó el perfil de amplificación de los marcadores *Inter Simple Sequence Repeat* (ISSR) en *Prosopis Juliflora* (Sw.) DC. y seleccionado aquellos con el perfil más adecuado. Para eso, fueron extraídos en el Laboratorio de Genética Aplicada y Molecular, de la Universidad Estadual del Sudoeste de Bahia, en Itapetinga-BA, el DNA genómico de 16 individuos recolectados en el Instituto Federal, Campus Itapetinga, Bahia. El perfil de amplificación se obtuvo utilizando 23 iniciadores ISSR, visualizados después de electroforesis en geles de agarosa al 2%, fotodocumentados bajo luz ultravioleta y luego se realizó el paso de genotipado. En total se observó amplificación de 206 marcadores, con un promedio de nueve marcas por iniciador, porcentaje polimórfico equivalente al 90% de los marcadores generados; contenido medio de información polimórfica de 0,63. Los valores medios de heterocigosidad esperada y observada fueron similares, con 0,28 y 0,29, respectivamente. Los resultados confirman que los iniciadores ISSR tienen características adecuadas para estudios genético-moleculares en *P. juliflora*, pudiendo destacarse entre los 23 iniciadores, 13 a priorizar (DiGA3'G, DiCA 3'RG, DiCA 3'YG, DiGA 3'C, DiGA 3'RC, DiGA 3'T, TriCAG 3'RC, TriTGT 3'YC, TriAAC 3'RC, TriAAG 3'RC, TriCGC 3'RC, TriGAC 3'RC e TriGGA 3'RC) en estudios genéticos de *P. juliflora*.

Palabras clave: ISSR; Polimorfismo; *Prosopis juliflora*; Transferibilidad.

1. Introduction

Mesquite (*Prosopis juliflora* Sw. DC.) (Linnaeus, 1753) (Fabaceae: Mimosoideae) (Ribaski et al., 2009; Burnett, 2017) is an allogamous, xerophytic evergreen, C3 plant (Pasicznik et al., 2004; Hussain et al., 2019). Its flowering occurs between the second and third year of life, from April, July, and September until November (Ribaski et al., 2009). Pollination is carried out mainly by *Appis mellifera* L. and *Trigona fuscipennis* Friese (De Sousa et al., 2016). Its fruits are yellowish indehiscent pods (ABD El Halim et al., 2015). For this species, dispersal and habitat are decisive for its survival (Yoda et al., 2015; De Souza Nascimento et al., 2020) and take place in different plant strata by animal grazing (mules, cattle, and camels) (Gonçalves et al., 2013; De Souza Nascimento et al., 2020; Al-Wardy et al., 2021).

Native to Mexico, Central America, and some South American countries (Nascimento et al., 2014), its efficiency in colonizing arid environments (Hussain et al., 2019) is mainly related to rapid growth and drought resistance (Yasuda et al., 2014; APG IV, 2016). Therefore, it has been introduced in countries with arid and semi-arid climate, such as Sudan, Sahel, South Africa, India, and Brazil (Beale et al., 2020). This species entered Brazil in 1942, 1946, and 1952 (Silva, 2000; BURNETT, 2017) due to its forage potential (pods) to feed herds in periods of grazing scarcity (Belmont et al., 2016).

In Brazil, mesquite is known as algaroba and is found from Northeast (Alagoas, Bahia, Ceará, Paraíba, Pernambuco, Piauí, Rio Grande do Norte States) to South (Rio Grande do Sul State) (Morim, 2015). It plays a major role for the country's economy (Ribaski et al., 2009; Burnett, 2017) as nutritional additive for animals (Aguiar et al., 2019; Khobondo et al., 2019), raw material for the pharmaceutical industry (Sharifi-Rad et al., 2019), human feeding (Lima et al., 2020; De Melo Cavalcante et al., 2022), production of bioethanol (De Melo Alves & Leite Filho, 2020) and alcoholic beverages (De Carvalho et al., 2020), and coal mining industry (CIRILO et al., 2021), as well as making of boards, stakes, and fences in farms (Rodrigues et

al., 2013).

Despite its several uses; little is known about mesquite genetic diversity. Molecular-genetic studies are essential for management and conservation strategies or breeding studies (Faleiro et al., 2018). Indeed, genetic characterization based on molecular markers includes defining a molecular marker to be used among available options, the Inter Simple Sequence Repeat (ISSR). These dominant inheritance markers can be used in any species, as previous genomic knowledge is needless, making them a promising tool for genetic studies of plant populations (Sarma & Tanti, 2017).

Besides defining markers, primers must also be selected. If previous characterizations do not exist or are insufficient, characterization and selection of primers becomes a priority step. As mesquite is essential for Northeastern Brazil development and genetic-molecular information on this species are scarce, this study aimed to characterize the amplification profile and select ISSR primers to support genetic studies in *P. juliflora*.

2. Methodology

Our research was carried out at the Applied Molecular Genetics Laboratory of the State University of Southwest Bahia (UESB, Itapetinga - Ba) and registered in the National System for Management of the Genetic Heritage and Traditional Knowledge Associated (SISGEN), under registration number AFF0E34. The botanical material was deposited in the Herbarium of the State University of Santa Cruz (UESC) under registration number: RG-14435. Leaf samples of *P. juliflora* were collected from the Federal Institute, Campus de Itapetinga, Bahia, at the geographic coordinates: 15°14'57.8" S latitude and 40°13'53.4" W longitude, in southwestern mesoregion of Bahia State (Brazil).

2.1 Genomic DNA Extraction, Purification, and Quantification

Genomic DNA extraction followed a protocol adapted and selected for the species (Santos et al., 2021a), according to method of Sunnucks and Halles (1996). First, 0.4 g young leaf tissue was macerated in 3.0 mL SDS buffer (sodium dodecyl sulfate), without liquid nitrogen and β - mercaptoethanol additions. Then, DNA was quantified using a 1% (w/v) agarose gel electrophoresis run, stained with 0.2% (w/v) Red Invitrogen Gel Biotium™. To do so, we used a series of known concentrations of Lambda phage DNA (Invitrogen™) for calibration. Afterwards, DNA purity was estimated by absorbance ratios A260/230 and A260/280, respectively, using BioDrop™ μ LITE (Whitehead Scientific). After obtaining genomic DNA concentrations, samples were standardized at 2 ng μ L⁻¹ and stored in a -20 °C freezer.

2.2 Amplification of Genetic Material

Polymerase chain reaction (PCR) was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™), using 23 ISSR primers (Table 1) in 16 *P. juliflora* samples. The PCR was prepared with 8 μ L 2 ng DNA, 1.7 μ L 10x PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 1.0 μ L MgCl₂, 1.0 μ L dNTP mix, 3.19 μ L Milli-Q water, 0.11 μ L Taq DNA polymerase (LGC biotechnology), and 1.0 μ L each primer, totaling a final volume of 16 μ L per reaction. The amplification program consisted of the following steps: initial denaturation at 95 °C for 5 min, followed by 34 cycles (94 °C for 50 s for denaturation, 48 °C for 60s for annealing, and 72 °C for 60 s for extension), and a final extension at 72 °C for 5 min.

2.3 Data Analysis

Amplification products were visualized in a 2% (m/v) agarose gel electrophoresis run with 5x TBE running solution for 120 minutes at 110 v. After electrophoresis, gels were analyzed in a transilluminator (Kodak) under ultraviolet light. The resulting images were used to classify primers as: (I) adequate, when the marker amplification profile is present in most

individuals; or (II) inadequate, when there were no tags or the marker amplification was limited to a few individuals.

Moreover, total number of markers and polymorphism percentage were estimated using the GenAlex 6.5 software (Smouse & Peakall, 2012). Expected heterozygosity (He) was obtained as proposed by Nei (1987), using the formula: $He = 1 - \sum p_i^2$; wherein p_i is the estimated allele frequency. Finally, polymorphic information content (PIC) values were calculated according to Botstein et al. (1980), as follows:

$$IC = 1 - \sum_{i=1}^n p_i^2 - \sum_{i=1}^n \sum_{j=1+1}^n 2p_i^2 p_j^2$$

wherein p_i is the frequency of the i -th allele of the studied locus.

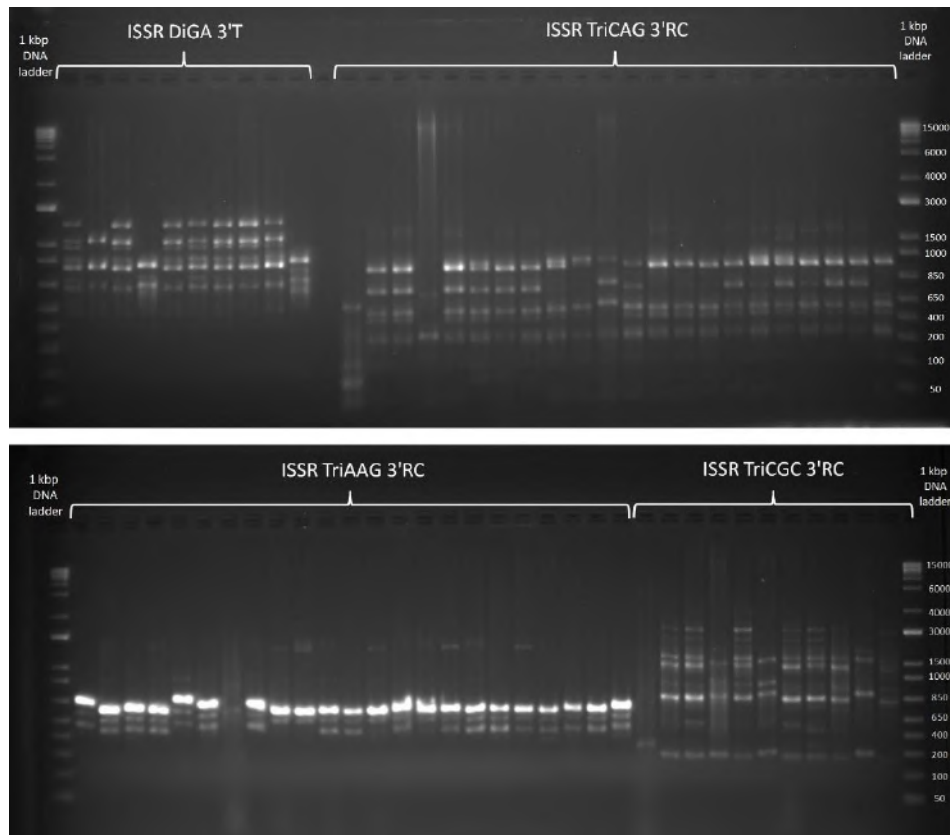
3. Results and Discussion

The 23 ISSR primers evaluated in the 16 *P. juliflora* individuals generated reproducible and easily scorable amplification profiles in the agarose gels. Thus, they were classified as adequate for the species (Figure 1).

From the amplification profiles, 206 markers were observed, averaging nine markers per primer used. The average polymorphism observed was 88.5%, ranging from 44.4% for the TriCAC'YC primer to 100% for the DiGA3'G, DiCA 3'RG, DiCA 3'YG, DiGA 3'T, TriCAC 3'RC, TriCAC 5'CY, TriCAG 3'RC, TriTGT 3'YC, TriAAC 3'RC, TriAAG 3'RC, TriTGG'RC, TriGAC 3'RC, and TriGGA 3'RC (Table 1). Although scarce, studies using ISSR markers in *P. juliflora* have also shown a high number of markers as we did in this study. While estimating similarity between *P. juliflora* (SW.) DC. and *P. cineraria* L. (Druce), Elmeer and Almalki (2011) identified 109 markers from 29 ISSR primers, an average below 4 markers per primer. These authors also observed a high polymorphism as in our study (88.5% on average), finding polymorphism in 108 of the 109 markers obtained.

By studying genetic diversity and gene flow in *P. cineraria*, Sharma et al. (2011) used the markers Simple Sequence Repeat (SSR) and Directed Amplification of Minisatellite-region DNA (DAMD) and also verified a high polymorphism percentage; such high polymorphism was found for 8 SSR markers and ranged from 75.0% to 100.0%, and an average of 92% was inferred for this marker. These polymorphism values found by these authors were higher than those obtained in our study. Furthermore, similar characterizations of *P. juliflora* made with primers analogous to resistance genes (RGA) have generated an average of 5.7 markers per primer (Santos et al., 2021b).

Figure 1 - Amplification profile of four Inter Simple Sequence Repeat primers (DiGA 3'T; TriCAG 3'RC; TriAAG 3'RC; TriCGC 3'RC) in samples of *Prosopis juliflora* (SW.) DC.



Source: Authors (2022).

Table 1 - Amplification profile characterization and genetic diversity index of *Prosopis juliflora* (Sw.) DC., using 23 ISSR (Inter Simple Sequence Repeat) primers.

Primer	Sequence	Total tags	% Polymorphism	He	PIC
DiGA3'G	(CA)8G	9	100.0	0.28	0.84
DiCA 3'RG	(CA)8RG	12	100.0	0.29	0.69
DiCA 3'YG	(CA)8YG	11	100.0	0.16	0.96
DiGA 3'C	(GA)8C	11	90.9	0.24	0.67
DiGA 3'RC	(GA)8RC	11	90.9	0.32	0.75
DiGA 3'T	(GA)8T	10	100.0	0.32	0.70
TriCAC 3'RC	(CAC)5RC	5	100.0	0.33	0.53
TriCAC 3'YC	(CAC)5YC	9	44.4	0.15	0.28
TriCAC 5'CY	CY(CAC)5	5	100.0	0.48	0.50
TriCAG 3'RC	(CAG)5RC	11	100.0	0.26	0.84
TriGTG 3'YC	(GTG)5YC	4	75.0	0.25	0.47
TriTGT 3'YC	(TGT)5YC	11	100.0	0.20	0.90
TriAAC 3'RC	(AAC)5RC	11	100.0	0.22	0.91
TriAAG 3'RC	(AAG)5RC	12	100.0	0.26	0.86
TriACA 3'RC	(ACA)5RC	6	66.6	0.30	0.44
TriAGA 3'RC	(ACG)5RC	7	71.4	0.17	0.45
TriTGG'RC	(CGC)5RC	8	100.0	0.34	0.50
TriCGA 3'RC	(GCA)5RC	5	60.0	0.22	0.30
TriCGC 3'RC	(GGA)5RC	12	90.9	0.34	0.68
TriGAC 3'RC	(GCA)5RC	10	100.0	0.32	0.56
TriGCA 3'RC	(GCA)5RC	8	75.0	0.29	0.52
TriGCC 3'RC	(CGC)5RC	7	71.4	0.37	0.46
TriGGA 3'RC	(GGA)5RC	11	100.0	0.35	0.75
Average		8.95	88.5	0.28	0.63

He= Expected heterozygosity; PIC= Polymorphic information content. Source: Authors (2022).

In this study, expected heterozygosity (He) for the 23 ISSR primers ranged from 0.15 (TriCAC primer 3'YC) to 0.48 (TriCAC primer 5'CY), averaging 0.28 (Table 1). Alves et al., (2014) developed and characterized 10 and 13 SSR primers for future genetic diversity studies in anthropized areas of South America. These authors observed He estimates for the genus *Prosopis* from 0.000 in *Prosopis rubriflora* Hassl. for Prb2 to 0.884 in *Prosopis ruscifolia* Gris for Prsc11. In Bolivia, Bessega et al., (2018) studied genetic diversity and differentiation in three *Prosopis alba* Griseb populations under different human disturbance levels and at different altitudes; they used 10 SSR microsatellites and observed that He ranged from 0.00 for GLP to 0.73 for GL12. As for Alves et al. (2014), variations in the species *P. juliflora* can be justified by anthropic pressures in areas where it is found, mainly cattle ranching, and/or its reproductive patterns. According to Zanella et al. (2012), the reproductive system of a target species should be known, as it can influence its population genetic variability.

In this study, mean PIC was 0.63, ranging from 0.28 (primer TriCAC 3'YC) to 0.96 (primer DiCA 3'YG). These findings are close to those of selection and characterization studies using microsatellite markers (SSR), such as those by Bessega et al. (2013) in *P. alba* (0.22 to 0.79) and *P. chilensis* (0.00 to 0.78) and by Alves et al. (2014) in *P. ruscifolia* (0.289 to 0.883) and *P. rubriflora* (0.073 to 0.791). Botstein et al. (1980) proposed that PIC values lower than 0.25 are poorly informative, between 0.25 and 0.50 moderately informative, and above 0.5 very informative; therefore, ISSR primers are

adequate and at least moderately informative. In this sense, 17 primers of the primers analyzed in this study can be classified as very informative, that is, with a PIC value ≥ 0.5 (Table 1). Moreover, for Muhammad et al. (2017), PIC variation depicts the diverse nature of genotypes.

Characterization and selection of primers have become increasingly frequent, including ISSR primers, as these are used in genetic studies for several native species, whose genome is still unknown (Sharma et al., 2011; Oliveira et al., 2017; Wang et al., 2017).

Given the importance of molecular markers and especially the scarcity of available primers, criteria and studies dedicated to the prior selection of primers should be prioritized. In this sense, some studies in the literature may be highlighted, for instance those in *Croton tetradenius* Baill (Almeida-Pereira et al., 2017) and *Zea mays* L. (Muhammad et al., 2017), as well as those on selection of markers analogous to resistance genes in *P. juliflora* (Santos et al., 2021b).

The polymorphic profile of ISSR primers observed in this study varied widely (88.5%). In this sense, Silva et al. (2018) described the importance of studies on amplification of ISSR markers, as monomorphic percentages may vary from one sample to another. Moreover, Santos et al. (2021b) proved the efficiency of RGA markers in a pioneering study on characterization and selection in *P. juliflora*. These authors reached He and PIC values from 0 to 0.38 and from 0 to 0.29, respectively. Although lower than our findings on He and PIC, of the 17 RGA primers tested by Santos et al. (2021b), 12 were highly polymorphic and can be used to investigate genetic variability in further studies on mesquite. Almeida-Pereira et al. (2017) also proved the effectiveness of ISSR markers to estimate genetic variability among accessions of the species *C. tetradenius*, which is endemic to northeastern Brazil.

Despite the naturally existing differences among the species and/or genera above mentioned, as well as among molecular marker classes, the averages of number of markers and percentage of polymorphism observed in *P. juliflora* denote that ISSR primers appear to be promising for different genetic studies for species belonging to the genus *Prosopis*.

Our amplification profile and PIC results attest to the efficiency of ISSR primers for future molecular analyses in *P. juliflora*, without changing amplification process, saving time and resources in developing new markers.

4. Conclusion

The ISSR markers show good transferability and a high percentage of polymorphism for the 16 individuals of mesquite (*P. juliflora*) considered in this study. Among the primers, 13 can be highlighted (DiGA3'G, DiCA 3'RG, DiCA 3'YG, DiGA 3' C, DiGA 3'RC, DiGA 3'T, TriCAG 3'RC, TriTGT 3'YC, TriAAC 3'RC, TriAAG 3'RC, TriCGC 3'RC, TriGAC 3'RC, and TriGGA 3'RC) for being adequate for genomic DNA amplification in the species, have polymorphic profile and can be classified as very informative.

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